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Nonclinical reproductive and developmental safety evaluation of the MAGE-A3 Cancer Immunotherapeutic, a therapeutic vaccine for cancer treatment

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ABSTRACT

We assessed potential toxic effects of the MAGE-A3 Cancer Immunotherapeutic on female fertility and embryo-fetal, pre- and post-natal development in rats and on male fertility in rats and monkeys. Three groups of 48 female (Study 1) or 22 male (Study 2) CD rats received 5 or 3 injections of 100 μL of saline, AS15 immunostimulant, or MAGE-A3 Cancer Immunotherapeutic (MAGE-A3 recombinant protein combined with AS15) at various timepoints pre- or post-mating. Male Cynomolgus monkeys (Study 3) received 8 injections of 500 μL of saline ($n=2$) or the MAGE-A3 Cancer Immunotherapeutic ($n=6$) every 2 weeks. Rats were sacrificed on gestation day 20 or lactation day 25 (Study 1) or 9 weeks after first injection (Study 2) and monkeys, 3 days or 8 weeks after last injection. Injections were well tolerated. Female rat mating performance or fertility, pre- and post-natal survival, offspring development up to 25 days of age, and male mating performance (rats) or fertility parameters (rats and monkeys) were unaffected.

1. Introduction

Chemotherapy has been a major approach in the treatment of cancer since the 1940s. Despite improvements in the survival rates, conventional chemotherapy has disadvantages, including limited cure rates and cytotoxicity to healthy cells that can sometimes result in severe or potentially life-threatening side effects [1–4]. Moreover, chemotherapy predominantly target rapidly dividing cells such as leukocytes and bone marrow precursors, and is considered immunosuppressive [4,5]. Furthermore, some patients do not respond to chemotherapy or develop resistance in the course of treatment [6,7].

Active cancer immunotherapy, used either alone or in combination with chemotherapy, offers a promising approach. The goal of cancer immunotherapy is to mobilize the patient's innate and adaptive immune systems to mount a response against cancer cells. Unlike chemotherapy, cancer immunotherapy more specifically targets tumor cells, decreasing the risk of damage to healthy tissues [4,5]. Although durable remissions of solid tumors have been obtained with antigen-nonspecific immunotherapeutic approaches, such as high-dose interleukin-2 (IL-2) or interferon-α, the associated toxicities or side effects remain high [8–10]. The goal of antigen-specific cancer immunotherapy is to elicit a *de novo* host immune response against selected tumor antigens using cancer immunotherapeutics [11–13]. The MAGE-A3 cancer immunotherapeutic, a therapeutic vaccine, was formulated with AS15, an immunostimulant containing 3-O-desacyl-4′-monophosphoryl lipid A (MPL), *Quillaja saponaria Molina*, fraction 21 (QS-21) and CpG in a liposome formulation. MPL is known to be a TLR4 agonist while CpG is able to bind to TLR9. CpG is also known to strengthen the anti-tumor cellular immunity. MPL and QS-21 act synergistically, being able to induce cell-mediated immune responses [14].

Abbreviations: LOQ, limit of quantification; HCD, Historical Control Data.

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The MAGE-A3 antigen represents an interesting target for immunotherapy, as it is expressed in various tumor types including melanoma and nonsmall cell lung cancer (NSCLC) [15–19]. In contrast, the human *MAGEA3* is silent in normal adult cells except in the germ cells of the testis, the trophoblasts of the placenta, and, at some stages, in the germ cells of fetal gonads (testis and ovary), where the lack of classical Class I (A, B, and C) and Class II human leukocyte antigen (HLA) expression should prevent antigen presentation [20–24].

Tumor antigens naturally trigger only weak spontaneous immunological responses, partly due to self-antigen tolerance [25]. In addition, antigens delivered as purified recombinant proteins are known to be poorly immunogenic and they need to be combined with immunostimulants to increase the magnitude of the response. MAGE-A3 Cancer Immunotherapeutic, containing the MAGE-A3 recombinant protein combined with the GSK proprietary immunostimulant AS15, is in Phase III clinical trials conducted by GSK for the treatment of MAGE-A3-expressing NSCLC (MAGRIT, NCT00480025) and melanoma (DERMA, NCT00796445). The results of the Phase II trials have been previously published [19,26].

Although cancer predominantly affects the elderly, a considerable proportion of patients is diagnosed at a younger age and may still wish to establish their families [27]. As survival rates following cancer treatment continue to improve, preservation of fertility has become an important issue for both the survivors and clinicians involved in cancer care. While it is well documented that chemotherapy drugs may cause male and female gonadal damage or dysfunction and infertility [28–30], little is known about potential toxic effects of active cancer immunotherapies. Indeed, given that *MAGEA3* is expressed in the testis, placenta, and at some stages in germ cells of embryonic gonads, assessment of the potential effects of repeated injections of MAGE-A3 Cancer Immunotherapeutic on embryo-fetal, pre- and post-natal development, and fertility is particularly important.

In support of the clinical development of MAGE-A3 Cancer Immunotherapeutic, we performed nonclinical safety studies evaluating potential toxicity of MAGE-A3 Cancer Immunotherapeutic to reproduction in two experimental animal models. Following the European Medicines Agency (EMA), U.S. Food and Drug Administration (FDA) and World Health Organization (WHO) guidelines [31–34], we evaluated potential toxic effects of MAGE-A3 Cancer Immunotherapeutic on female fertility and embryo-fetal and pre- and post-natal development in rats (Study 1), on male fertility in rats (Study 2) and on male fertility endpoints in Cynomolgus monkeys (Study 3). The rat was chosen as the test species because of the requirements for a rodent species by regulatory agencies, and is the most widely used species for pre- and post-natal developmental toxicity assessments. In this species, the MAGE-A3-related protein Exhibits 45% of identity with the human protein (sequence coverage of 83.8%) [35]. The Sprague-Dawley Crl:CD® strain was chosen as it generally known to exhibit robust immune responses. The choice of this species was also based on a preliminary pilot study that showed the induction of MAGE-A3-specific antibodies in rats after an immunization schedule and dosing (1/5th of the human dose) of the adjuvanted MAGE-A3 cancer immunotherapeutic similar to the one applied in the reprotoxicity study. In addition, Cynomolgus monkey was used as an animal model in Study 3 because the monkey MAGE-A3-related protein is highly homologous (>95% identity) to the human MAGE-A3 protein [35] and is expressed in the monkey testis and in cells at the same differentiation stage as in the human. In addition, the monkey was chosen as pharmacologically relevant species because it was demonstrated in previous studies that this species mounted antibody responses to MAGE-A3 at clinically meaningful doses [36]. Both species were proposed and accepted by regulatory authorities.

2. Materials and methods

2.1. Regulatory considerations and ethical statement

The studies were conducted in compliance with the EMA ICH Harmonized Tripartite Guideline for Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility [32], taking into account the FDA requirements for reproductive studies on vaccines, the Note for guidance on preclinical pharmacological and toxicological testing of vaccines (CPMP/SWP/465/95) [33], WHO guidelines on Nonclinical Evaluation of Vaccines [34] and the FDA Guidance for Industry [31].

The studies were conducted in accordance with Good Laboratory Practice (GLP) standards, including the UK GLP regulations (Act 1986) [37], The Organization for Economic Co-operation and Development (OECD) Principles on Good Laboratory Practice [38], Directive 2004 of the European Parliament and of the council [39], Arrêté du 14 mars 2000 relatif aux bonnes pratiques de laboratoire du Ministère de l'Emploi et de la Solidarité [40], and the Huntingdon Life Sciences (HLS; Eye, UK; Studies 1 and 2; rats) or CiToxLAB (Evreux, France; Study 3; monkeys) standard operating procedures. Each laboratory had AAALAC accreditation and an in-house Ethical Review Panel (ERP). The CiToxLAB Ethical Committee reviewed the study plan before the initiation of the study in monkeys. For studies 1 and 2 in rats, the HLS ERP reviewed the Project License Protocol to ensure that the procedures stated within the Project License comply with regulatory guideline requirements.

2.2. Study design

Studies were conducted under GLP conditions at the HLS laboratories (Studies 1 and 2) or at the CiToxLAB laboratories (Study 3**)**. Serology evaluations were performed under non-GLP conditions at GSK laboratories (Rixensart, Belgium).

In Study 1, 144 of 156 (16 rats with body weight below the range specified in the protocol were discarded) female rats were allocated to three groups (1:1:1) of 48 rats that received an initial intramuscular (IM) injection of 100 μL of saline (control group), AS15 immunostimulant (AS15 group), or the MAGE-A3 Cancer Immunotherapeutic (MAGE-A3 recombinant protein combined with the AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic group) 28 days before pairing (initial injection) (Fig. 1A). The dosing regimen was established based on the results from a preliminary pilot study which showed that following the administration of a single dose of 1/5th (100 μL) of the human dose of MAGE-A3 Cancer Immunotherapeutic at day −28 before mating, seroconversion occurred at day −5 before mating. Forty-four females from each group with positive signs of mating were retained and received additional injections on days 6, 8, 11 and 15 after mating; the remaining rats were killed six days after pairing/separation. Of the 44 rats retained in each group, 22 were killed on gestation day 20 (embryo-fetal phase) and the remaining 22 animals were allowed to rear their young to day 25 of age (littering phase) (Fig. 1A). The offspring did not receive any injections.

In Study 2, 147 rats, 74 males and 73 females, were weighed and divided into body weight strata (5 g range); 132 sexually mature rats, 66 males and 66 females, were allocated to three groups (1:1:1) of 44 rats (each comprising 22 males and 22 females), by selecting animals from each body weight range in rotation, after animals with atypical body weight had been discarded. This procedure ensured that all groups contained populations of rats with similar initial mean and range of body weights. To cover the whole period of spermatogenesis, male rats received IM injections of 100 μL of saline (control group), AS15 (AS15 group) or MAGE-A3 Cancer Immunotherapeutic (MAGE-A3 recombinant protein

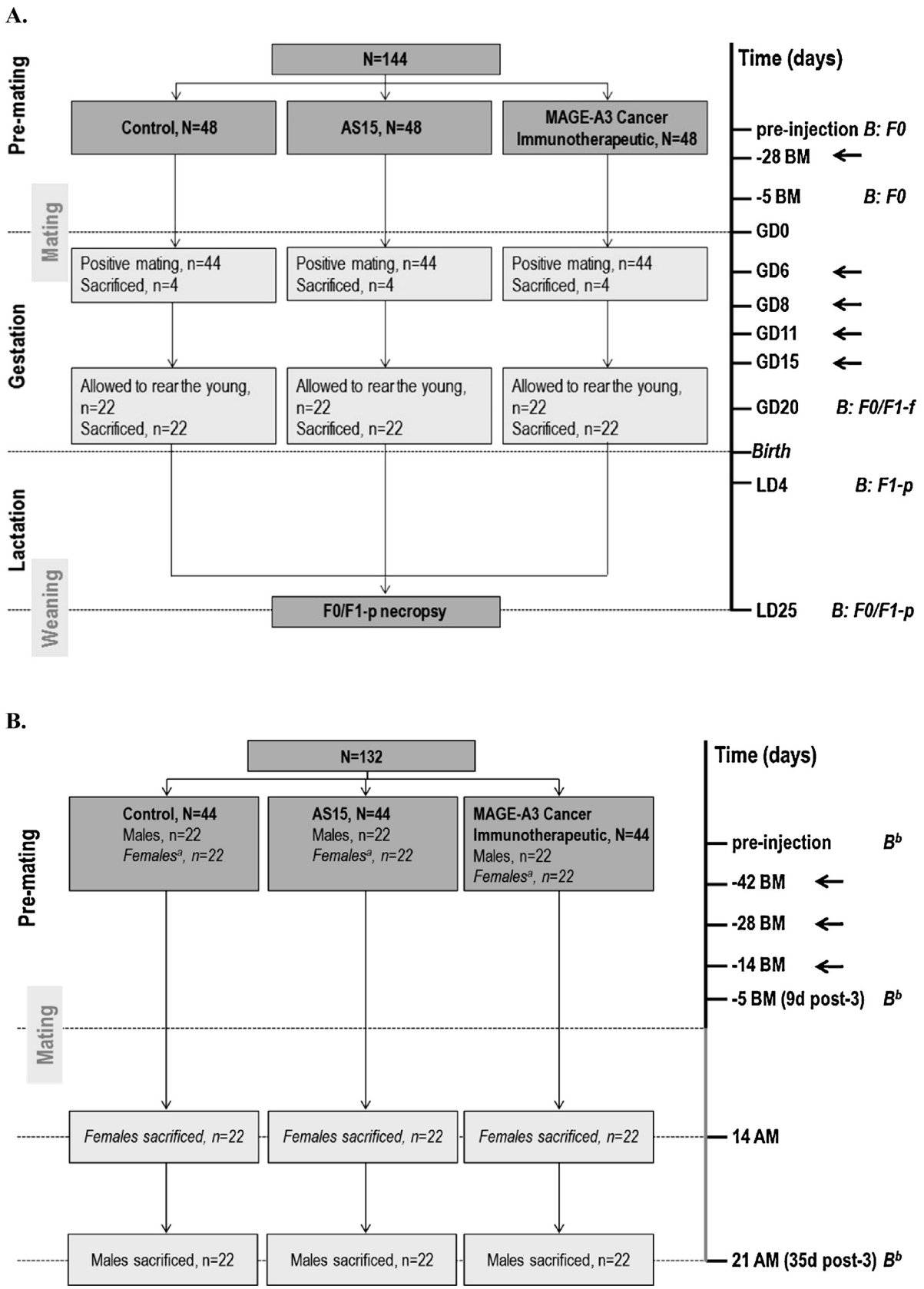

Fig. 1. Study design. (A) Study 1 and (B) Study 2. Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 recombinant protein combined with AS15; *N*, total number of rats per group; *n*, number of rats within the group; B, blood sampling; F0, F0 females; F1-p, offspring; F1-f, fetuses; BM, before mating; GD, gestation day; LD, lactation day; AM, after mating; 9d post-3, 9 days post 3rd injection; 35d post-3, 35 days post 3rd injection (termination). The arrows indicate the timepoints at which rats received the injections of saline, AS15 immunostimulant or MAGE-A3 Cancer Immunotherapeutic.

[a] Females were untreated.

[b] Males only.

combined with the AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic group) on days 42, 28 and 14 before pairing; females were not treated. After the treatment period, males were paired on a one-to-one basis with untreated females for a period of up to three weeks. Females were sacrificed 14 days after mating, and males at least nine weeks after the first injection (Fig. 1B).

In Study 3, eight of nine sexually mature male monkeys were selected for the study, based on clinical and laboratory examinations, and manually allocated randomly to two groups that received an IM injection of 500 μL of saline ($n = 2$; control group) or MAGE-A3 Cancer Immunotherapeutic ($n = 6$; MAGE-A3 recombinant protein combined with the AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic group) every two weeks, with a total of 8 injections. One monkey from the control group and four from the MAGE-A3 Cancer Immunotherapeutic group (early sacrifice group animals) were sacrificed three days after the last injection (day 102); the remaining monkeys (one control, two MAGE-A3 Cancer Immunotherapeutic; recovery group animals) were sacrificed after an eight-week treatment-free period.

2.3. Study objectives

The studies assessed potential toxic effects of the injections of MAGE-A3 Cancer Immunotherapeutic on female CD rat fertility and embryo-fetal, pre-natal and early post-natal development (Study 1), male fertility and early embryonic development in CD rats (Study 2), and male fertility endpoints in Cynomolgus monkeys (Study 3; reversibility of potential toxic effects over a period of eight weeks after the last injection was also assessed). In addition, immune responses (serology) to MAGE-A3 Cancer Immunotherapeutic were evaluated under non-GLP conditions in all studies.

2.4. Test items

One dose of the MAGE-A3 Cancer Immunotherapeutic consisted of 300 μg recombinant MAGE-A3 antigen (ProteinD-MAGE-A3-His; recMAGE-A3) [41] and a fixed dose of the AS15 immunostimulant, containing MPL (GSK Vaccines, Rixensart, Belgium), QS-21 (Antigenics Inc, a wholly owned subsidiary of Agenus Inc., Lexington, MA, USA), CpG 7909 synthetic oligodeoxynucleotides containing unmethylated CpG motifs, and liposome (50 μg MPL, 50 μg QS-21 and 420 μg CpG 7909). The control was a 0.9% sterile sodium chloride solution (saline).

2.5. Treatment and administration

In all studies, the IM route was selected to mimic the intended route of administration in human therapeutic use.

Rats received 100 μl injections of saline, AS15, or the MAGE-A3 Cancer Immunotherapeutic (MAGE-A3 recombinant protein combined with AS15) administered in the anterior left or right thigh muscle. Each dose of the MAGE-A3 Cancer Immunotherapeutic corresponded to 1/5th of the human dose (500 μL), considered to be the maximal volume to be given as a single IM injection to a rat, representing an over-exposure of approximately 50–70 times the levels that humans are exposed to in a single dose, based on a 50–70 kg human and 170–250 g rat.

Monkeys received 500 μl injections of saline or the MAGE-A3 Cancer Immunotherapeutic. Each dose of the MAGE-A3 Cancer Immunotherapeutic corresponded to a full human dose and represented approximately 10- to 20-fold the human dose relative to the body weight, based on a 70 kg human and a 3.5–7.5 kg monkey. Injections were administered in different muscles at the different timepoints, whereby some muscles received only a single injection and others up to two injections (Table 1), allowing for evaluation of the appearance of lesions over time and comparison between sites receiving one or two injections.

Table 1
Injection sites and schedule in Study 3 (monkeys).

Site	Day							
	1[a]	15	29	43	57	71	85	99
1: Right *biceps brachii*^{S}	x							
2: Left *biceps brachii*^{R}		x			x			
3: Left front thigh^R			x			x		
4: Left back thigh^R				x			x	
5: Right front thigh^S								x

S, single injection site; R, repeated injection site.
[a] First injection.

2.6. Experimental animals, housing and husbandry

2.6.1. Studies in rats

Crl:CD® Sprague-Dawley (SD) IGS BR rats were received from Charles River Laboratories (UK).

In Study 1, female rats were approximately 7 weeks old and with weights ranging from 167 to 231 g at the study start, and were 11–12 weeks old at the time of mating. Six days after mating, females allocated to treatment during gestation weighed from 257 to 378 g.

In Study 2, male rats were approximately 8–9 weeks old at the study start and had weights ranging from 276 to 300 g, while the untreated female rats were approximately 10–11 weeks old with body weights ranging from 226 to 250 g. The males allocated to the study groups were approximately 9–10 weeks old and had weights ranging from 332 to 400 g, and the females on gestation day 0 were approximately 11–12 weeks old with body weights ranging from 239 to 280 g.

The housing conditions were previously described [42]. Briefly, the rats were allowed free access to a standard rodent diet with no added antibiotic or other chemotherapeutic or prophylactic agent.

2.6.2. Study 3 (monkeys)

Nine male, purpose-bred Cynomolgus monkeys (*Macaca fascicularis*) were received from Noveprim Europe (Ebene, Mauritius). At the study start, monkeys allocated to the study were 43–51 months old with a mean body weight of 5.4 kg, considered as sexually mature (based on testicular volume, semen sampling, and testosterone serum levels). During the study period, monkeys were housed in individual stainless steel cages, at 22 (±3) °C with relative 50% (±30%) humidity in a 12 h/12 h light/dark cycle. Monkeys received approximately 180 g/day of OWM (E) SQC SHORT expanded diet (Dietex France, SDS, Saint Gratien, France) and a daily fruit supplement. All monkeys had free access to tap water.

2.7. Clinical signs

Rats were inspected visually at least twice daily for signs of ill-health or reaction to treatment. In addition, a more detailed physical assessment of general health was performed on each rat in Study 1 weekly before pairing, on days 0, 7, 14 and 20 after mating, and on days 1, 7, 14, 21 and 25 of lactation, and in Study 2, weekly on males, and on days 0, 7 and 14 after mating on females. Local reactions at the injection sites were assessed for four days following each injection, then weekly, on day 0 of gestation (Study 1 only) and at termination.

Monkeys were assessed for mortality, morbidity and clinical signs twice a day.

2.8. Body weight

In Study 1, body weight of each F0 female rat was recorded on the first day of treatment (day −28), weekly until mating was detected, on days 0, 3, 6, 8, 11, 15, 17 and 20 after mating, and on days 1, 4, 7, 11, 14, 18, 21 and 25 of lactation.

In Study 2, male rats were weighed at the beginning of the study and twice weekly until termination. Females were weighed on days 0, 4, 7, 11 and 14 after mating.

In Study 3, the body weight of each monkey was recorded twice before the beginning of the treatment period, on the first day of treatment and once a week until the end of the study, except in weeks 13 and 22, when the body weight was recorded twice a week, and in week 14, when the body weight was not recorded.

2.9. Food consumption

In Study 1, food consumption was recorded for the following periods: weekly before pairing, days 0–2, 3–5, 6–7, 8–10, 11–14, 15–16 and 17–19 after mating, and days 1–3, 4–6, 7–10, 11–13, 14–17, 18–20 and 21–24 of lactation. The mean daily consumption (g/rat/day) was calculated for each animal.

In Study 2, food consumption was recorded twice weekly from the start of treatment until the animals were paired for mating. From these records, the mean daily consumption per animal (g/rat/day) was calculated for each phase and for each cage.

In Study 3, the amount of food consumed by each monkey was checked daily over 21 days before the beginning of the treatment period and then throughout the study.

2.10. Mating (studies in rats)

Females were paired on a one-to-one basis on day 28 after the pre-mating injection in Study 1 and 42 days following the first pre-pairing immunization in males, for a period of up to 3 weeks in Study 2. In both studies, during the mating period, 1 male and 1 female rat were housed together per cage. Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepared from each female and examined for the presence of spermatozoa and the stage of the estrous cycle. The day on which mating was detected was designated day 0 of gestation. Once mating occurred, the males and females were separated and smearing was discontinued. The mating percentage and conception rate were assessed later.

2.11. Cesarean section, uterine/ovary evaluations and fetal examinations

In Study 1, six days after mating, or after the required number of rats was allocated to gestation, four rats per group were sacrificed. Of the 44 rats in each group, 22 per group (21 in the AS15 group; one dead female) were sacrificed on day 20 after mating. Fetuses were sacrificed by chilling on a cold plate. For females allowed to litter, the litter size was standardized to ten on day 4 of age by randomized culling. The females and the remaining offspring were sacrificed on day 25 of lactation. All adult rats and all offspring aged ≥14 days were sacrificed by CO_2 asphyxiation, while offspring aged <14 days were sacrificed by intraperitoneal injection of sodium pentobarbitone. Four-day-old offspring selected for blood sampling were sacrificed by decapitation. Macroscopic pathology examinations of the females, fetuses, and offspring were performed as previously described [42].

For each female, the number of corpora lutea in each ovary and the number of implantation sites, the number and distribution of resorption sites and live and dead fetuses were recorded for each uterine horn. All fetuses and placentae were dissected from the uterus and weighed individually. Fetuses were individually identified within the litter, using a coding system based on their position in the uterus. Each fetus and placenta was externally examined and any abnormalities were recorded. In addition, the sex of each fetus was recorded. The fetuses received a fresh external examination, and were assessed internally post-fixation. Approximately half of the fetuses in each litter were subject to gross internal examination of the viscera of the neck, thorax and abdominal cavities. These fetuses were then eviscerated and their skeletons were fixed in Industrial Methylated Spirit, prior to processing and staining with Alizarin Red. The stained fetuses were assessed for skeletal development and abnormalities. The remaining fetuses were fixed whole in Bouin's fluid, then free-hand serial sections were prepared and examined under the microscope and visceral abnormalities were assessed. Normally rare abnormalities which were definitely detrimental to normal subsequent development or could even be lethal, such as ventricular septal defects, were defined as major abnormalities. Minor differences from normal that are detected relatively frequently or are considered to have little detrimental effect and may be a transient stage in the development, such as a bipartite centrum or a dilated ureter, were defined as minor differences.

In Study 2, the untreated females were sacrificed on day 14 after mating, and the following reproductive assessments were made: the number of *corpora lutea* in each ovary and the number of implantation sites, the number and distribution of resorption sites, and the number of live and dead embryos. All adult rats were sacrificed by CO_2 asphyxiation and embryos by decapitation.

2.12. Parturition and post-natal observations (Study 1)

Parturition and post-natal observations, and the duration of gestation were evaluated as previously described [42]. From day 20 after mating, females were inspected three times daily for evidence of parturition. The progress and completion of parturition was monitored, and numbers of live and dead offspring were recorded as well as any observed difficulties. All litters were examined at approximately 24 h after birth (day 1 of age), and then daily thereafter. Clinical signs and litter size were recorded daily, the sex ratio of each litter was recorded on days 1, 4 and 25 of age, and individual offspring body weights were recorded on days 1, 4, 7, 11, 14, 18, 21 and 25 of age.

The following pre-weaning reflex developmental tests were performed on each offspring: surface righting (assessed daily from day 2 of age until achieved), air righting (assessed daily from day 16 until day 21 of age), auditory function (startle response to a sudden sharp sound, assessed on day 20 of age), and pupillary reflex (pupil closure response of dark adapted eyes to a bright point source of light, assessed on day 20 of age).

Offspring surviving to day 25 of age were subject to a complete macroscopic examination; any retained tissues were checked before disposal of the carcass.

2.13. Evaluation of spermatogenesis

In Study 2, immediately after the scheduled sacrifice, samples for sperm analysis (motility, morphology and sperm count) were taken. Sperm count and motility were analyzed using the Hamilton Thorne IVOS Computer Assisted sperm Analyzer version 12.3c. The left vas deferens, epididymides and testes were removed and the epididymides and testes were weighed.

In Study 3, semen samples were taken by electroejaculation from all monkeys twice during the pre-treatment period, approximately every three weeks (±three days) during the treatment period and three times during the treatment-free period using a constant voltage stimulator (6020 Stimulator, Harvard Apparatus) according to a procedure approved by the CITOxLab Ethical

Committee. Monkeys were not anesthetized for this procedure. Samples were microscopically analyzed for the presence, motility, morphology, and count of spermatozoa. To assess the effects of the treatment with the MAGE-A3 Cancer Immunotherapeutic on the gonads and prostate, measurements of the testes and prostate volume were performed on all monkeys twice during the pre-treatment period, approximately every three weeks (±three days) during the treatment period and three times during the treatment-free period. Prior to examination, monkeys were anesthetized by an IM injection of ketamine hydrochloride (Imalgene® 500, Merial, France) in the right calf muscle. The volume of each testis and of the prostate was measured by ultrasonography. The measurements were performed using a Megas CV probe (ELEKTO GBM, Laval, France).

2.14. Blood sampling

In Study 1, blood samples were obtained from the females at pre-treatment, at day −5 (before pairing), at day 20 after mating (embryo-fetal phase), and day 25 of lactation (littering phase). Samples were collected into plain tubes either from the sublingual vein or from the retro-orbital sinus. In Study 2, blood samples were obtained from the males at pre-treatment (five days before pairing) and at termination. Samples were collected into plain tubes from the sublingual vein during the in-life phase and from the retro-orbital sinus at termination. Each rat was held under isoflurane anesthesia during the sampling procedure.

In Study 3, monkeys were not anesthetized before blood sampling and no prior fasting was required, unless other investigations were planned on the same day. For the assessment of serology parameters, approximately 3 ml of venous blood were taken from the cephalic or saphenous vein into a plain tube once during pre-treatment and on completion of the treatment and treatment-free periods. For the assessment of testosterone levels, approximately 1 ml of blood was collected in tubes without anticoagulant in the morning (usually at 8 a.m.) twice during the pre-treatment period and then once every three weeks (±three days) during the treatment period. Testosterone levels were assessed using a direct radioimmunological method (RIA); each analysis was processed in duplicate.

2.15. MAGE-A3-specific antibody analysis

MAGE-A3-specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Individual antibody responses were measured and reported either individually or as geometric mean titers (GMTs). In Study 1, the sera of fetuses were analyzed individually or as a pool. The recMAGE-A3 protein was used as coating antigen for Studies 1 and 2, while a recombinant MAGE-A3 protein produced in baculovirus was used for Study 3. The limit of quantification (LOQ) of ELISA was the lowest titer calculated in the range of quantification (20–80%). The LOQ was calculated for each plate and was between 10 and 38 ELISA units (EU)/ml (Study 1), between 22 and 32 EU/ml (Study 2) or between 287 and 350 EU/ml (Study 3). A titer value of a sample inferior to the LOQ was fixed at 24 EU/ml (Study 1) or 27 EU/ml (Study 2); these values corresponded to the average of the lower and upper LOQ values for each study.

2.16. Sacrifice

In Study 2, males were sacrificed by CO_2 asphyxiation at least nine weeks after the first injection, following completion of the necropsy of the females.

In Study 3, after completion of the treatment or treatment-free period, after at least 14 h of fasting, monkeys were tranquilized by an IM injection of ketamine hydrochloride, anesthetized by an IV injection of thiopental sodium and sacrificed by exsanguination.

2.17. Necropsy; tissue collection, processing and examination

A full macroscopic examination of all tissues was performed on all adult rats and monkeys. The injection site reactions were recorded. Abnormalities in the appearance or size of organs and tissues were recorded and the required tissue samples preserved in an appropriate fixative.

In Studies 2 and 3, the following organs were weighed: epididymides, seminal vesicles (rats only), prostate, testes, and pituitary gland (monkeys only). Bilateral organs were weighed individually. In Study 2, the right epididymis and right testis were fixed in Bouin's solution prior to transfer to 70% industrial methylated spirit. The prostate and seminal vesicles were preserved in 10% neutral buffered formalin. In Study 3, the organs were preserved in 10% buffered formalin (pituitary gland, prostate and seminal vesicles) or in modified Davidson's fixative (testes and epididymides). All collected tissues were embedded in paraffin wax, sectioned at a thickness of approximately 4 microns and stained with hematoxylin eosin and Periodic acid Schiff (PAS, testis only). All the preserved tissues were examined for all animals sacrificed on completion of the treatment period. Microscopic examination of the testes was performed after the treatment periods in both studies to evaluate the stages of maturation of the spermatic cells.

2.18. Immunohistochemistry

Immunohistochemistry evaluations of the expression pattern of the human MAGE-A3 or the monkey and rat MAGE-A3-related protein in the testes were performed in non-GLP conditions before the studies reported herein were initiated. Immunohistochemistry data obtained partly supported the choice of the animal species.

Paraffin sections (5 μm thick) of the human (obtained from the National Disease Research Interchange, Philadelphia, US), Cynomolgus monkey (Noveprim Ltd. – Port Louis, Mauritius) and Sprague Dawley rat (Charles River Laboratories, Lyon, France) testes were incubated with a rabbit polyclonal antibody (TA242; 1/1000) raised against the human recMAGE-A3 protein, or with TA242 pre-incubated with 47 μg/ml recMAGE-A3 protein. Antibody binding was detected using a biotinylated anti-rabbit mouse monoclonal antibody (Sigma) and streptavidin-horseradish conjugate (Invitrogen). 3,3′-Diaminobenzidine was used as chromogen (DAB detection kit, Invitrogen). Slides were counterstained with Mayer's Hemalum (Klinipath).

2.19. Statistics (studies in rats)

Statistical analyses were performed where there was indication of possible meaningful intergroup differences. In Study 1, all statistical analyses were carried out using the individual animal (or means in the case of the litter) as the basic experimental unit. For Study 2, all statistical analyses were carried out separately for males and females. Data relating to food consumption were analyzed on a cage basis.

The following sequence of statistical tests was used for body weight, food consumption, placental, litter and fetal weights, litter size and survival indices, pre-weaning examinations data, organ weights, sperm analysis, motility and count: if 75% of the data (across all groups) were the same value, then a frequency analysis was applied. Treatment groups were compared using pairwise Fisher's Exact tests [43] for each dose group against the control. If Bartlett's test for variance homogeneity [44] was not significant at the 1% level, then treatment groups were compared using Student's *t*-test. If Bartlett's test was significant at the 1%

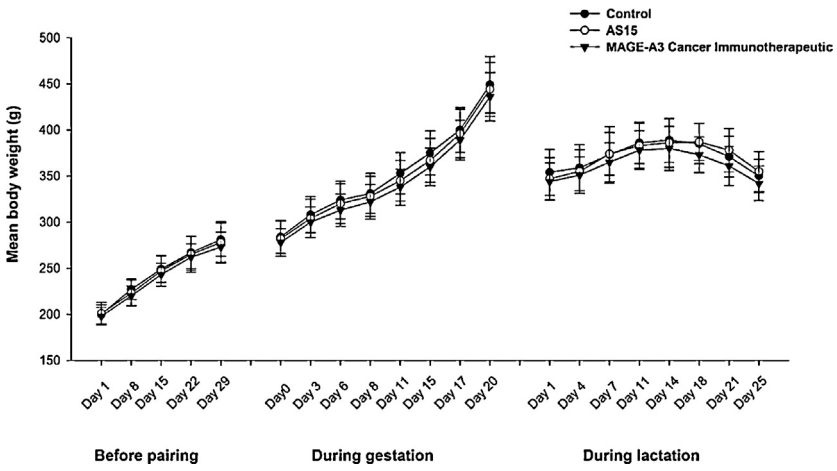

Fig. 2. Body weight of female rats in Study 1. Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic. $N = 48$ rats in each group before pairing; $N = 44$ rats in the control group and $N = 42$ rats in AS15 and MAGE-A3 Cancer Immunotherapeutic group during gestation; $N = 22$ rats in the control group and $N = 21$ rats in AS15 and MAGE-A3 Cancer Immunotherapeutic group during lactation.

level, logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then treatment groups were compared using a Wilcoxon rank sum test [45].

Pre- and post-implantation loss and sex ratio were analyzed by generalized mixed linear model with binomial errors, a logit link function and litter as a random effect [46]. Each treated group was compared to control using a Wald Chi-square test.

For resorptions, each treated group was compared to control by exact Wilcoxon rank sum test [45].

Comparisons between AS15 or MAGE-A3 Cancer Immunotherapeutic groups and the control group were performed for the percentage of litters where a specific abnormality was detected (Study 1); Fisher's exact test was used for each of the comparison. Overall percentage of fetuses affected by a specific abnormality was analyzed also and the analysis provided a similar conclusion. These results should be interpreted with caution since multiple comparisons were made.

For organ weight data (Study 2), analysis of covariance was initially performed using terminal body weight as covariate. If the within-group relationship between organ weight and body weight was significant at the 10% level [47], then the treatment comparisons were made on adjusted group means in order to allow for differences in body weight which might influence the organ weights.

Due to the small sample size and the high inter-individual variability expected for the evaluated parameters, no statistical analyses were performed in Study 3.

3. Results

3.1. Study 1: Effects of MAGE-A3 Cancer Immunotherapeutic on female rat fertility, embryo-fetal and pre- and post-natal development

3.1.1. Mortality, clinical observations, body weight and food consumption

There was one unexpected death during the study; following the last injection, one female from the AS15 group was sacrificed for welfare reasons on day 18 of gestation due to swollen areas on the upper ventral thorax, ventral abdomen and perigenital area. The swelling of the perigenital region caused displacement of the vagina. Macroscopic examination revealed large, irregular subcutaneous masses in the left and right mammary areas extending around the vaginal and anal openings; the cut-surface revealed firm, pale nodular tissue. This event was considered incidental and unrelated to treatment with AS15, as it occurred in a single female.

In general, routine external examinations during the course of the study showed no remarkable findings. Among animals receiving the MAGE-A3 Cancer Immunotherapeutic, nineteen animals showed limited use of the left hind limb on Day 2 of the pre-mating phase. This finding was considered to be related to the administration of the initial immunization, and did not persist. During the gestation phase, several animals receiving either the MAGE-A3 Cancer Immunotherapeutic or AS15 were observed to have a swollen area on the right sacral region. Two days following the pre-mating injection of the MAGE-A3 Cancer Immunotherapeutic, six of 48 rats had swellings around the injection site that persisted for up to four days; swelling was not observed following injections of AS15 alone or saline. During the gestation phase, swelling around the injection site and, to a lesser extent, edema, were seen in rats receiving AS15 or the MAGE-A3 Cancer Immunotherapeutic. The number of rats in which swelling was recorded was highest 7–18 days after mating, coinciding with a 1- to 4-day period following each injection. During the lactation phase, swelling around the injection site was observed on day 1 of lactation in one control rat and in two rats from the AS15 group.

There was no effect of MAGE-A3 Cancer Immunotherapeutic or AS15 injections on body weight or food consumption during the study (Fig. 2).

3.1.2. Reproductive assessment

All but one (AS15 group) female allocated to the littering phase became pregnant. All but one (MAGE-A3 Cancer Immunotherapeutic group) female gave birth to live offspring. There was no effect of the treatment with the MAGE-A3 Cancer Immunotherapeutic or AS15 on the parturition process or on gestation length. Gestational length was 22–23 days, except for one control female (24 days) (Table 2).

All females surviving to scheduled termination on day 20 after mating were pregnant. Maternal treatment with the MAGE-A3 Cancer Immunotherapeutic or AS15 had no effect on the mean numbers of corpora lutea, implantations, embryo-fetal resorptions, live young or pre- and post-implantation losses (Table 3). In the AS15 group, the mean number of male fetuses was statistically significantly lower (6.9 vs 8.9; $p<0.01$) and the mean number of female fetuses was statistically significantly higher (8.9 vs 7.3; $p<0.05$) than in the control group (Table 3). This difference was considered to have occurred by chance and to be unrelated to treatment,

Table 2
Mating performance and gestation length (Study 1).

	Control	AS15	MAGE-A3 Cancer Immunotherapeutic
Number of female rats/cage			
Acclimatization period	Up to 4	Up to 4	Up to 4
Mating period	1[a]	1[a]	1[a]
Gestation period (up to Day 17 after mating)	1	1	1
Littering period (from Day 17 after mating to Day 25 of lactation)	1 + litter	1 + litter	1 + litter
Number mating, *n* (%)	47 (98)	48 (100)	47 (98)
Number pregnant, *n* (%)	47 (100)	47 (98)	45 (96)
Number pregnant killed on Day 20, *n* (%)	22 (100)	22 (100)	21 (95)
Number littering live pups, *n*	22	21	21
Gestation length, *n* (%)			
22 days	9 (41)	8 (38)	7 (33)[b]
22.5 days	6 (27)	7 (33)	7 (33)[b]
23 days	6 (27)	6 (29)	7 (33)[b]
23.5 days	0	0	0
24 days	1 (5)	0	0

Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic; *n*, number of rats; %, percentage.

[a] Besides 1 female rat per cage 1 male rat was housed in the cage.

[b] Percentage distribution of gestation lengths calculated from 21 animals (one pregnant female failed to litter).

because a similar pattern was not replicated in the subset of litters allocated to the littering phase, where the mean numbers of male or female offspring were similar (see Section 3.1.3.). Group mean values for placental, litter and fetal weights were similar for all groups.

The incidence of major abnormalities (including misshapen lens, cleft palate, membranous ventricular septal defect, malrotated heart, inguinal hernia, imperforate anus and bent scapula) in fetuses was similarly low in all groups, with four of 356 (1.1%) affected fetuses in the control group and two of 334 (0.6%) affected fetuses in the MAGE-A3 Cancer Immunotherapeutic group (no affected fetuses were reported in the AS15 group). There was no difference in the fetal and litter incidence of minor abnormalities in the AS15 and MAGE-A3 Cancer Immunotherapeutic groups compared to the control group, except for the litter incidence of umbilical left artery abnormalities, which was statistically significantly higher ($p = 0.0485$) in the MAGE-A3 Cancer Immunotherapeutic group than in the control group (Table 4). However, this minor visceral finding was regarded as a normal variation as the incidence was within the Historical Control Data (HCD) range.

In the AS15 group there was a slightly higher incidence of fetuses/litters with the following minor abnormalities compared with the saline group (Table 2): sutural bone abnormalities, cervical ribs abnormalities, incomplete ossification of cranial centers and sacrocaudal vertebrae, and ossified cervical vertebral centra. The incidence of sutural bone and sacrocaudal vertebrae abnormalities were within the HCD range. The incidences of ossified cervical vertebral centra were within the fetal incidence HCD range, but outside the litter incidence HCD range. The low incidences of fetuses/litters with cervical ribs abnormalities and incomplete ossification of cranial centers were just outside the HCD range. In the MAGE-A3 Cancer Immunotherapeutic group, a number of differences from the saline group were similar to those recorded in the AS15 group: there were slightly higher incidences of fetuses/litters with cervical ribs abnormalities and incomplete ossification of cervical and sacrocaudal vertebrae. All the litter incidences were within the HCD range and were thus regarded as a normal variation.

Injections of AS15 or the MAGE-A3 Cancer Immunotherapeutic did not affect mating performance or fertility. Of the 142 females that mated, 139 attained pregnancy. Two females, one in the control group and one in the MAGE-A3 Cancer Immunotherapeutic

Table 3
Litter data – group mean values for female rats killed on Day 20 of gestation (Study 1).

	Control (*N* = 22)	AS15 (*N* = 21)	MAGE-A3 Cancer Immunotherapeutic (*N* = 21)
Corpora lutea, mean (SD)	18.0 (1.62)	17.3 (2.35)	17.6 (2.31)
Implantations, mean (SD)	16.7 (1.70)	16.5 (2.04)	16.7 (2.58)
Resorptions, mean	0.5	0.8	0.8
Live young, mean (SD)			
Total	16.2 (1.87)	15.8 (2.34)	15.9 (2.32)
Male	8.9 (2.44)	6.9[**] (2.05)	8.2 (2.07)
Female	7.3 (2.53)	8.9[*] (2.01)	7.7 (2.22)
Sex ratio, % male	55.0	43.7[**]	51.9[‡]
Implantation loss, %			
Pre	7.0	5.8	5.7
Post	3.3	4.8	4.3
Placental weight (g), mean (SD)	0.53 (0.048)	0.55 (0.071)	0.53 (0.052)
Fetal weight (g), mean (SD)	3.89 (0.230)	3.90 (0.171)	3.83 (0.215)
Male fetal weight (g), mean (SD)	3.97 (0.235)	4.02 (0.221)	3.90 (0.232)
Female fetal weight (g), mean (SD)	3.79 (0.240)	3.81 (0.157)	3.75 (0.218)

Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic; *N*, number of rats in the group; SD, standard deviation; %, percentage; Pre, pre implantation phase (losses due to nonfertilization of ova); Post, post implantation phase.

[*] Statistically different value compared to the control group ($p < 0.05$).

[**] Statistically different value compared to the control group ($p < 0.01$).

[‡] Statistically different value compared to the AS15 group ($p < 0.05$).

Table 4
Incidence of minor visceral and skeletal abnormalities in fetuses and litters (Study 1).

Group	Fetuses			Litters		
	Control	AS15	MAGE-A3 Cancer Immunotherapeutic	Control	AS15	MAGE-A3 Cancer Immunotherapeutic
Visceral abnormalities						
Number of examined rats	179	165	168	22	21	21
Left umbilical artery, *n*	–	3	4	–	3	4*
Skeletal abnormalities						
Number of examined rats	177	166	166	22	21	21
Cervical rib, *n*	1	5	4	1	5	3
Incomplete ossification/unossified						
Cranial centers, *n*	25	36	24	10	14	12
Sacrocaudal vertebrae, *n*	6	9	10	4	4	7
Precocious ossification						
Cervical vertebral centra, *n*	3	8	1	3	6	1

Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic; *n*, number of affected rats.
* Statistically significantly higher value as compared to control (Fisher's exact test $p = 0.0485$).

group, failed to mate prior to the cessation of pairing; neither female showed normal estrous activity during the pairing period. Given that only two females, one of which was from the control group, failed to mate, a relation to treatment with MAGE-A3 Cancer Immunotherapeutic was excluded.

3.1.3. F1 litter responses

There was no effect of maternal treatment with the MAGE-A3 Cancer Immunotherapeutic or AS15 on mean implantation counts, litter size or offspring survival (in-utero or pre-weaning), or body weight (Table 5). The sex ratio, as assessed by the percentage of males and females per litter, was comparable in all groups and in line with expectations.

The mean age at attainment for the surface (ability to regain a normal upright stance when placed in a supine position) and air (ability to fall down on its feet when dropped in a supine position) righting reflexes for offspring was similar between groups (Table 5). There was no effect of maternal treatment on the percentage of offspring showing auditory and pupillary responses on day 20 of age.

3.1.4. Post-mortem examinations

Macroscopic examination of females at scheduled termination did not indicate any effect of treatment with either the MAGE-A3 Cancer Immunotherapeutic or AS15.

Of the few offspring that died during the early post-natal period, most showed an absence of milk in the stomach, which is common in offspring that die at an early age. There were no significant findings at *post-mortem* examination for offspring that survived to the scheduled termination.

Table 5
Litter size, offspring survival, body weight and developmental parameters.

	Control (*N* = 22)	AS15 (*N* = 21)	MAGE-A3 Cancer Immunotherapeutic (*N* = 21)
Implantations, mean (SD)	16.6 (3.5)	17.5 (1.2)	16.4 (2.0)
Total litter size, mean (SD)	15.4 (3.5)	16.8 (1.4)	15.3* (3.1)
Post implantation survival index, %	91.8	95.1	93.1
Live birth index, %	98.8	98.0	98.4
Preculling viability index %	97.3	99.4	98.1
Postculling viability index, %	95.9	100	95.8
Body weight, male/female offspring			
Before cull			
Day of age 1, mean (SD)	7.3 (0.8)/6.9 (0.7)	7.0 (0.5)/6.5** (0.5)	7.0 (0.6)/6.7 (0.7)
Day of age 4, mean (SD)	10.4 (1.5)/9.9 (1.3)	9.9 (0.9)/9.2** (1.0)	10.4 (1.2)/10.0* (1.3)
After cull			
Day of age 4, mean (SD)	10.4 (1.4)/10.0 (1.2)	9.9 (0.9)/9.2** (1.0)	10.4 (1.2)/10.0* (1.3)
Day of age 7, mean (SD)	16.7 (2.0)/15.8 (2.0)	16.2 (1.0)/15.2 (1.4)	16.9 (2.1)/16.3* (2.0)
Day of age 11, mean (SD)	26.9 (2.7)/25.9 (3.1)	26.6 (1.6)/25.1 (2.1)	27.2 (2.8)/26.7 (3.0)
Day of age 14, mean (SD)	34.8 (2.4)/33.2 (3.0)	34.2 (2.1)/32.5 (2.8)	34.6 (3.3)/33.9 (3.4)
Day of age 18, mean (SD)	44.5 (3.5)/42.5 (3.8)	43.4 (2.9)/41.6 (3.5)	43.8 (4.2)/43.1 (4.4)
Day of age 21, mean (SD)	56.0 (4.5)/53.5 (4.5)	53.9 (3.5)/51.3 (4.6)	54.0 (7.0)/53.0 (6.2)
Day of age 25, mean (SD)	79.1 (5.3)/74.2 (5.0)	76.6 (4.6)/71.4 (5.3)	75.9 (9.4)/73.0 (6.9)
Pre-weaning examinations			
Surface righting			
Day of age, mean (SD)	3.6 (0.74)	3.8 (0.61)	3.5 (0.74)
Air righting			
Day of age, mean (SD)	17.0 (0.44)	17.0 (0.47)	17.1 (0.99)
	Control (*N* = 210)	**AS15 (*N* = 210)**	**MAGE-A3 Cancer Immunotherapeutic (*N* = 194)**
Pupil reflex, *n*, (%) pass	210 (100.0)	210 (100.0)	192 (99.0)
Startle response, *n* (%) pass	210 (100.0)	210 (100.0)	194 (100.0)

Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic; *N*, number of tested rats; *n*, number of affected rats; %, percentage; SD, standard deviation. Live birth index (%) = (Number live offspring on Day 1 after littering/Total number of offspring born) × 100; Preculling viability index (%) = (Number live offspring on Day 4 before culling/Number live offspring on Day 1 after littering) × 100; Postculling viability index (%) = (Number live offspring on Day 21 after culling/Number live offspring on Day 4 after culling) × 100.
* Statistically different value compared to the AS15 group ($p < 0.05$).
** Statistically different value compared to the control group ($p < 0.05$).

Table 6
Body weight change in male rats given as difference between the measure timepoint and Day 1 (Study 2).

Group		Days 1–4	Days 1–8	Days 1–11	Days 1–15	Days 1–18	Days 1–22	Days 1–25	Days 1–29	Days 1–32	Days 1–36	Days 1–39	Days 1–43	Days 1–46	Days 1–50	Days 1–53	Days 1–57	Days 1–60	Days 1–64
1	Mean (g)	19	34	46	60	72	84	93	103	109	127	126	136	139	147	155	159	167	173
	SD	4.5	7.7	9.1	11.5	13.2	15.2	16.6	18.0	19.8	21.4	21.2	23.2	24.4	24.9	25.0	25.4	28.1	27.2
2	Mean (g)	15*	30	43	61	70	84	94	108	113	125	130	142	141	149	156	161	167	173
	SD	5.5	8.8	8.9	11.2	12.2	12.5	13.1	14.8	15.3	18.5	16.0	20.6	24.3	24.0	26.3	26.3	26.9	26.6
3	Mean (g)	16*	33	46	62	70	85	94	107	112	125	131	142	143	151	162	167	175	180
	SD	4.9	7.0	10.4	13.3	14.5	16.6	18.0	20.0	21.5	22.1	22.6	24.9	24.1	25.6	26.2	27.0	26.8	27.8

Group 1, rats injected with 100 μL of saline; Group 2, rats injected with 100 μL of AS15 immunostimulant; Group 3, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic; SD, standard deviation.
* Significant difference as compared to the control group ($p < 0.05$). $N = 22$ rats in each study group.

3.1.5. Serology

No recMAGE-A3-specific antibodies were detected in the sera of rats before the first injection (day −35) except for one of 44 females from the MAGE-A3 Cancer Immunotherapeutic group, in which a very low titer (75 EU/ml), close to the LOQ, was detected. No recMAGE-A3-specific antibodies were detected in the sera of the dams, fetuses or offspring of the control group (except for one of 11 fetuses for which a titer of 2886 EU/ml was measured at day 20 of gestation).

In the AS15 group, recMAGE-A3-specific antibodies were detected in the dams at only one timepoint (gestation day 20; five days after the fifth injection) at which 15 of 21 dams (57%) had a very low, but measurable GMT, according to the LOQ definition used. This GMT was 500-fold lower compared to the GMT of rats following five injections of MAGE-A3 Cancer Immunotherapeutic (46 EU/ml vs 29,443 EU/ml) (Supplementary Fig. 1A). This observation was transient as recMAGE-A3-specific antibodies were not detected 28 days later (at day 25 of lactation; 33 days after the fifth injection). No recMAGE-A3-specific antibodies were detected in the sera of fetuses or offspring from the AS15 group (Supplementary Fig. 1B and C), with the exception of one of 72 four-day-old offspring and two of 84 25-day-old offspring with very low antibody levels detected (54, 34 and 66 EU/ml, respectively).

In the MAGE-A3 Cancer Immunotherapeutic group, recMAGE-A3-specific antibodies were detected 23 days following the first injection (five days before mating) in all but one dam, with a GMT markedly higher as compared to baseline (3014 EU/ml). After five injections, recMAGE-A3-specific antibody titers further increased in all dams from this group, with GMTs of 29,443 EU/ml at gestation day 20 (five days after the fifth injection) and 21,079 EU/ml at day 25 of lactation (33 days after the fifth injection) (Supplementary Fig. 1A). recMAGE-A3-specific antibodies were also detected in the sera of fetuses and offspring, with GMTs of 4217 EU/ml for the fetuses at gestation day 20, and 7567 EU/ml and 8850 EU/ml, respectively at four, and 25 days of age for the offspring (Supplementary Fig. 1B and C).

3.2. Study 2: Effects of MAGE-A3 Cancer Immunotherapeutic on rat male fertility and early embryonic development

3.2.1. Mortality, clinical observations, body weight and food consumption

No unscheduled mortalities or signs of ill-health were recorded. Injections of AS15 or the MAGE-A3 Cancer Immunotherapeutic were well tolerated.

Injection site reactions, restricted to swollen areas around the injection site, were generally observed 1–4 days after each injection of the MAGE-A3 Cancer Immunotherapeutic or AS15.

Following the first injection of the MAGE-A3 Cancer Immunotherapeutic or AS15, mean body weight gain during the following three days was slightly but statistically significantly lower in males from these groups than in the control group (mean values: 15 g in AS15 and 16 g in the MAGE-A3 Cancer Immunotherapeutic vs 19 g in the control group; $p < 0.05$). Thereafter, mean body weight gains were similar to control (Table 6). There were no intergroup differences in the mean body weight performance of the untreated females during gestation (data not shown).

There was no effect of the MAGE-A3 Cancer Immunotherapeutic or AS15 injections on the mean food consumption of the male rats prior to pairing.

3.2.2. Mating performance and fertility

There was no effect of the MAGE-A3 Cancer Immunotherapeutic or AS15 injections on mating performance or fertility (Table 7). All males successfully mated within the first four days of the pairing

Table 7
Litter data – group mean values for female rats killed on day 14 of gestation (Study 2).

Number of rats per cage	Males	Females
Pre-mating period	Up to 4	Up to 4
Mating period	1	1
Gestation	–	Up to 4[a]
Males after mating	Up to 4	–

	Control (*N* = 22)	AS15 (*N* = 22)	MAGE-A3 Cancer Immunotherapeutic (*N* = 21)[b]
Mating males, *n* (%)	22 (100)	22 (100)	22 (100)
Number achieving pregnancy, *n* (%)	22 (100)	22 (100)	22 (100)
Fertility index, %	100	100	100
Corpora lutea, mean (SD)	16.5 (2.24)	17.1 (1.44)	17.5 (1.71)
Implantations, mean (SD)	15.5 (2.02)	16.0 (2.05)	16.4 (1.80)
Resorptions, mean	1.2	0.5	0.9
Live embryos, mean (SD)	14.3 (3.40)	15.5 (2.44)	15.6 (2.31)
Implantation loss, %			
Pre	5.8	6.0	6.1
Post	8.2	3.6	5.4

Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic; *N*, number of rats in the group; *n*, number of rats affected; SD, standard deviation; %, percentage; Pre, pre implantation phase (losses due to nonfertilization of ova); Post, post implantation phase.
[a] Females at the same gestation phase.
[b] The number of rats included in the analyses was *N* = 21, except for the evaluation of the mean number of *corpora lutea* and mating performance and fertility, which was *N* = 22.

period and all untreated females sustained pregnancy through mid-gestation.

3.2.3. Reproductive assessment

There was no effect of treatment of males with the MAGE-A3 Cancer Immunotherapeutic or AS15 on the litter, as assessed by the mean number of implantations, resorptions, live embryos and pre- and post-implantation losses (Table 7). All untreated females were pregnant with live embryos on day 14 of gestation.

There was no effect of treatment with the MAGE-A3 Cancer Immunotherapeutic or AS15 on sperm motility (Table 8). When compared with the control group, treated males showed a marginal but statistically significant increase in the mean percentage of motile sperm (94% in the AS15 group and 95% in the MAGE-A3 Cancer Immunotherapeutic group vs 91% in the control group; $p < 0.05$). However, this difference was partially attributable to one control male with a particularly low percentage motile sperm (65%). When compared with the historical data range for males of the same strain and of a similar age (±two weeks), all mean values for percentage of motile sperm were considered to be within a normal range. Therefore, a relationship to treatment with the MAGE-A3 Cancer Immunotherapeutic or AS15 was excluded.

There was no effect of treatment with MAGE-A3 Cancer Immunotherapeutic or AS15 on testis weight, the numbers of homogenization-resistant spermatids, or the numbers or concentration of spermatozoa in the cauda epididymis.

Microscopic examination did not reveal any effect of treatment with the MAGE-A3 Cancer Immunotherapeutic or AS15 on sperm morphology, which was normal in over 97% of the sperm samples in each group.

3.2.4. Macropathology and histopathology

Following necropsy, there were no major macroscopic findings. There was no effect of the MAGE-A3 Cancer Immunotherapeutic or AS15 injections on mean weight of the testes, epididymides, seminal vesicles or prostate.

Microscopic examination of the right testis and epididymis, prostate and seminal vesicles did not reveal any changes that could be associated with injections of MAGE-A3 Cancer Immunotherapeutic or AS15. Evaluation of the seminiferous tubules of the right testis revealed no cell- or stage-specific abnormalities.

3.2.5. Serology

No recMAGE-A3-specific antibodies were detected in the sera of rats from the three groups before the first injection or in the

Table 8
Reproductive parameters of male rats given in group mean values (*N* = 22 for each group) – Study 2.

	Control	AS15	MAGE-A3 Cancer Immunotherapeutic
Motile sperm, % (SD)	91 (7)	94* (4)	95** (5)
Progressively motile sperm, % (SD)	56 (13)	51 (13)	58 (14)
Cauda epididymis			
Weight, g (SD)	0.250 (0.024)	0.253 (0.023)	0.250 (0.028)
Sperm count, millions/g (SD)	1077 (300)	970 (239)	1077 (302)
Total, million (SD)	268 (79)	245 (64)	269 (78)
Testis			
Weight, g (SD)	1.84 (0.15)	1.75 (0.14)	1.81 (0.19)
Sperm count, millions/g (SD)	174 (52)	168 (52)	174 (66)
Total, million (SD)	320 (102)	295 (93)	314 (119)

Control, rats injected with 100 μL of saline; AS15, rats injected with 100 μL of AS15 immunostimulant; MAGE-A3 Cancer Immunotherapeutic, rats injected with 100 μL of MAGE-A3 Cancer Immunotherapeutic; SD, standard deviation; %, percentage.
* Statistically different value compared to the control group ($p < 0.05$).
** Statistically different value compared to the control group ($p < 0.01$).

Table 9
Seminological evaluation of sperm samples from the control and MAGE-A3 Cancer Immunotherapeutic treated monkeys (Study 3).

Group	Parameter	Treatment period (day)						Recovery period (day)		
		−19	−5	21	43	63	91	105	126	147
Sperm count (number of spermatozoa 10 × 6/mL)										
Control	*N*	2	2	1	2	2	2	1	1	1
	Mean (SD)	1140(681)	1089(323)	757 (NA)	1673(265)	1569(560)	1318(67)	1172 (NA)	1860 (NA)	1365 (NA)
MAGE-A3 Cancer Immunotherapeutic	*N*	4	6	2	4	5	3	2	2	2
	Mean (SD)	977(310)	960(461)	780 (69)	1527(546)	2073(739)	1392(193)	1441 (168)	1527 (749)	943 (173)
Sperm motility (percentage of motile spermatozoas)										
Control	*N*	2	2	2	2	2	2	1	1	1
	Mean % (SD)	100(0.0)	100(0.0)	100 (0.0)	100 (0.0)	100 (0.0)	100(0.0)	100 (NA)	100 (NA)	100 (NA)
MAGE-A3 Cancer Immunotherapeutic	*N*	6	6	4	5	5	5	2	2	2
	Mean % (SD)	100(0.0)	100(0.0)	75.0 (50.0)	100 (0.0)	100 (0.0)	100(0.0)	100 (0.0)	100 (0.0)	100 (0.0)
Sperm morphology (percentage of normally shaped spermatozoas)										
Control	*N*	2	2	1	2	2	2	1	1	1
	Mean % (SD)	88.5 (2.1)	80.5 (0.7)	48.0 (NA)	85.0 (4.2)	83.5 (19.1)	74.0 (5.7)	59.0 (NA)	80.0 (NA)	85.0 (NA)
MAGE-A3 Cancer Immunotherapeutic	*N*	6	6	2	4	5	5	2	2	2
	Mean % (SD)	90.7 (3.2)	80.0 (4.7)	58.0 (2.8)	85.5 (2.6)	84.6 (5.5)	88.2 (6.2)	78.5 (7.8)	87.5 (2.1)	89.5 (0.7)

Control, monkeys injected with 500 μL of saline; MAGE-A3 Cancer Immunotherapeutic, monkeys injected with 500 μL of MAGE-A3 Cancer Immunotherapeutic; *N*, number of assessed monkeys; SD, standard deviation; mean %, mean value of the % of motile or normally shaped spermatozoa after evaluation of N monkeys; NA, not applicable.

sera of the control rats at any timepoint. Following the third injection to rats from the AS15 group, a very low GMT (31 EU/ml), in the range of the LOQ, was detected in eight of 22 rats. In contrast, following the last injection of MAGE-A3 Cancer Immunotherapeutic, recMAGE-A3-specific antibody titers were markedly increased (GMT = 17,075 EU/ml) as compared to baseline, and were still detected at necropsy (at nine weeks following the first injection), although at lower levels (GMT = 6734 EU/ml) (Supplementary Fig. 2).

3.3. Study 3: Potential toxic effects of the MAGE-A3 Cancer Immunotherapeutic on the reproductive organs of male monkeys and reversibility of the potential toxic effects over a treatment-free period of eight weeks

3.3.1. Mortality, clinical observations and body weight

IM administrations of the MAGE-A3 Cancer Immunotherapeutic were well tolerated and only incidental and unrelated findings were observed. There were no serious reactions to treatment and no mortality. All monkeys maintained or gained body weight over the period of the study, and food consumption was unaffected by treatment with MAGE-A3 Cancer Immunotherapeutic.

3.3.2. Reproductive assessment

Regular monitoring of testicular and prostatic volume (see 2.13), seminology parameters (see 2.13) and circulating testosterone levels (see 2.14) was conducted throughout the study (during the treatment and treatment-free periods).

Testicular volume and prostate volume, as estimated by echography, showed a tendency to increase slightly over the period of the study (treatment period and successive treatment-free period). There was no indication of any effect of treatment with MAGE-A3 Cancer Immunotherapeutic on these parameters.

Sperm count and morphology were unaffected by treatment with the MAGE-A3 Cancer Immunotherapeutic; sperm motility was uniformly high in the control and the MAGE-A3 Cancer Immunotherapeutic-treated monkeys throughout the study (Table 9). Occasional sperm samples could not be evaluated due to sample coagulation or insufficient volume; however, these events were unrelated to treatment. It was thus concluded that repeated IM administrations of MAGE-A3 Cancer Immunotherapeutic did not affect seminological parameters.

Circulating testosterone levels were characterized by substantial variability but there was no indication that treatment with the MAGE-A3 Cancer Immunotherapeutic had any effect on testosterone levels during the treatment or treatment-free periods (Supplementary Table 1). The observed variations were attributed to normal physiological fluctuations, i.e. pulsatile secretion of testosterone in males [48,49].

3.3.3. Pathology findings

No evidence of treatment-related changes on the weights of testes, prostate, epididymis or the pituitary gland was found at the end of the treatment or treatment-free periods (Table 10). The control monkey was the biggest, hence the most mature and therefore had higher absolute organ weights. However, the relative-to-body organ weights were close or within the range of those observed in the vaccinated monkeys. Also, when a careful microscopic examination was performed on the testes, no morphological changes in the association of various germ cell types at a specific time-point of development of the seminiferous tubule were observed. Multinucleated germ cells were sporadically seen in 1 (of 4) treated monkeys and in the control individual. Atrophy of some seminiferous tubules was recorded in 1 (of 4) treated monkeys. This finding was characterized by foci of seminiferous tubules with germ cell depletion and Sertoli cell vacuolation. As the microscopic findings

Table 10
Reproductive organ weights at necropsy (Study 3).

Organ, mean values	Control[a]		MAGE-A3 Cancer Immunotherapeutic	
	K0 (*N* = 1)	R1 (*N* = 1)	K0 (*N* = 4)	R1 (*N* = 2)
Final body weight, g (SD)	7900.0	5640.0	[4.700–7.420]	[5.280–6.400]
Epididymis left				
Weight, g (SD)	3.97	3.36	[2.118–2.752]	[2.563–3.075]
% Body (SD)	0.05024	0.05961	[0.037–0.056]	[0.040–0.058]
Epididymis right				
Weight, g (SD)	4.08	3.06	[2.141–2.930]	[2.615–3.066]
% Body (SD)	0.05166	0.05429	[0.039–0.054]	[0.041–0.058]
Pituitary gland				
Weight, g (SD)	0.06300	0.06000	[0.059–0.078]	[0.055–0.095]
% Body (SD)	0.00080	0.00106	[0.001–0.002]	[0.001–0.002]
Prostate				
Weight, g (SD)	3.51	2.53	[2.677–5.358]	[2.289–2.476]
% Body (SD)	0.04441	0.04488	[0.049–0.086]	[0.036–0.047]
Testis left				
Weight, g (SD)	21.35	16.26	[14.43–19.71]	[12.05–17.33]
% Body (SD)	0.27020	0.28830	[0.264–0.313]	[0.188–0.328]
Testis right				
Weight, g (SD)	21.04	15.68	[13.831–20.667]	[11.717–16.910]
% Body (SD)	0.26638	0.27801	[0.253–0.332]	[0.183–0.320]

Control, monkeys injected with 500 μL of saline; MAGE-A3 Cancer Immunotherapeutic, monkeys injected with 500 μL of MAGE-A3 Cancer Immunotherapeutic; K0, terminal sacrifice group; R1, recovery/post-treatment group; *N*, number of monkeys in the group; SD, standard deviation; %, percentage.
[a] Due to the limited number of monkeys in the control group, no statistical analysis was performed on the organ weight.

were limited to those spontaneously observed in male Cynomolgus monkeys of this age (and were each present in one animal only) a relationship to treatment was excluded.

In summary, the weights of epididymides, pituitary, prostate and testes were found to be unaffected by treatment with MAGE-A3 Cancer Immunotherapeutic. On histopathological evaluation of the epididymides, pituitary gland, prostate, seminal vesicles and testes, findings were limited to those spontaneously observed in Cynomolgus monkeys of this age.

3.3.4. Serology

Antibodies specific for the recombinant MAGE-A3 protein (bacMAGE-A3) were not detected in monkeys included in this study before MAGE-A3 Cancer Immunotherapeutic injection or in the control group at any timepoint.

Three days following the last injection of MAGE-A3 Cancer Immunotherapeutic, all monkeys ($n = 6$) demonstrated high MAGE-A3-specific antibody titers that were still detectable eight weeks after the last injection (for the two monkeys evaluated), although at lower levels (8896 EU/ml and 18,344 EU/ml) than three days after the last injection (21,088–51,946 EU/ml) (Supplementary Fig. 3).

3.4. Detection of MAGE-A3 in the testis

The MAGE-A3 expression pattern was evaluated in the testes of rats, Cynomolgus monkeys and humans by immunohistochemistry using TA242 antibody. A strong staining limited to the germ cells was observed in all three species as shown in rat (Fig. 3A), monkey (Fig. 3C), and human (Fig. 3E) testes. However, in the rat, the staining was observed in cells at a later stage of differentiation (late spermatids) compared to the monkey and the human samples (spermatogoniae and primary spermatocytes). No staining was observed when the antibody was pre-incubated with the human recMAGE-A3 protein before use (Fig. 3B, D and F), suggesting that the staining observed in rat and monkey samples was specific to the rat or monkey MAGE-A3-related protein.

4. Discussion

The MAGE-A3 Cancer Immunotherapeutic is currently under evaluation in two Phase III clinical trials in patients with NSCLC or melanoma. As MAGE-A3 is specifically expressed by tumors, the risk of mounting an active immune response against normal tissues is considerably reduced. In healthy adult organs, MAGE-A3 expression is only found in the germ cells of the testes and in the placenta; however, these cells, being devoid of HLA Class I and Class II molecules, have no antigen presentation capabilities, excluding the possibility of developing immune-related toxicity [23,24,50]. Moreover, the MAGE-A3 antigen is not expressed at the surface of the cells, but only in their cytoplasm [51]. As a consequence, MAGE-A3-specific antibodies cannot access the antigen directly and cell killing via an antibody-mediated mechanism, such as the antibody-dependent cellular cytotoxicity, cannot occur. The MAGE-A3-specific T-cells can recognize antigen expressing cells only if peptides derived from MAGE-A3 are presented in the context of HLA Class I–II. If such presentation does not occur due to the lack of HLA Class I and II expression (like in germ cells or spermatozoid in the testis), the antigen expressing cells could not be recognized by the MAGE-A3-specific T-cells that the immunotherapeutic may have induced. Similarly to humans, the germ cells of the fetal gonads are considered immune privileged sites in all animal species due to the absence of major histocompatibility complex molecules and are thus protected from immune attack. Nevertheless, potential toxic effects on the reproductive functions via other mechanisms as well as potential effects on embryo-fetal development cannot be excluded.

Therefore, to support the clinical development of the MAGE-A3 Cancer Immunotherapeutic, we evaluated the potential toxic effects induced by injections of the MAGE-A3 Cancer Immunotherapeutic on embryo-fetal, pre- and post-natal development in female rats and on male fertility in rats and monkeys. The present studies did not evaluate the morphology of the germ cells from the fetal gonads; the assessment of their functionality would have required the extension of the postnatal period into adulthood up to a mating trial.

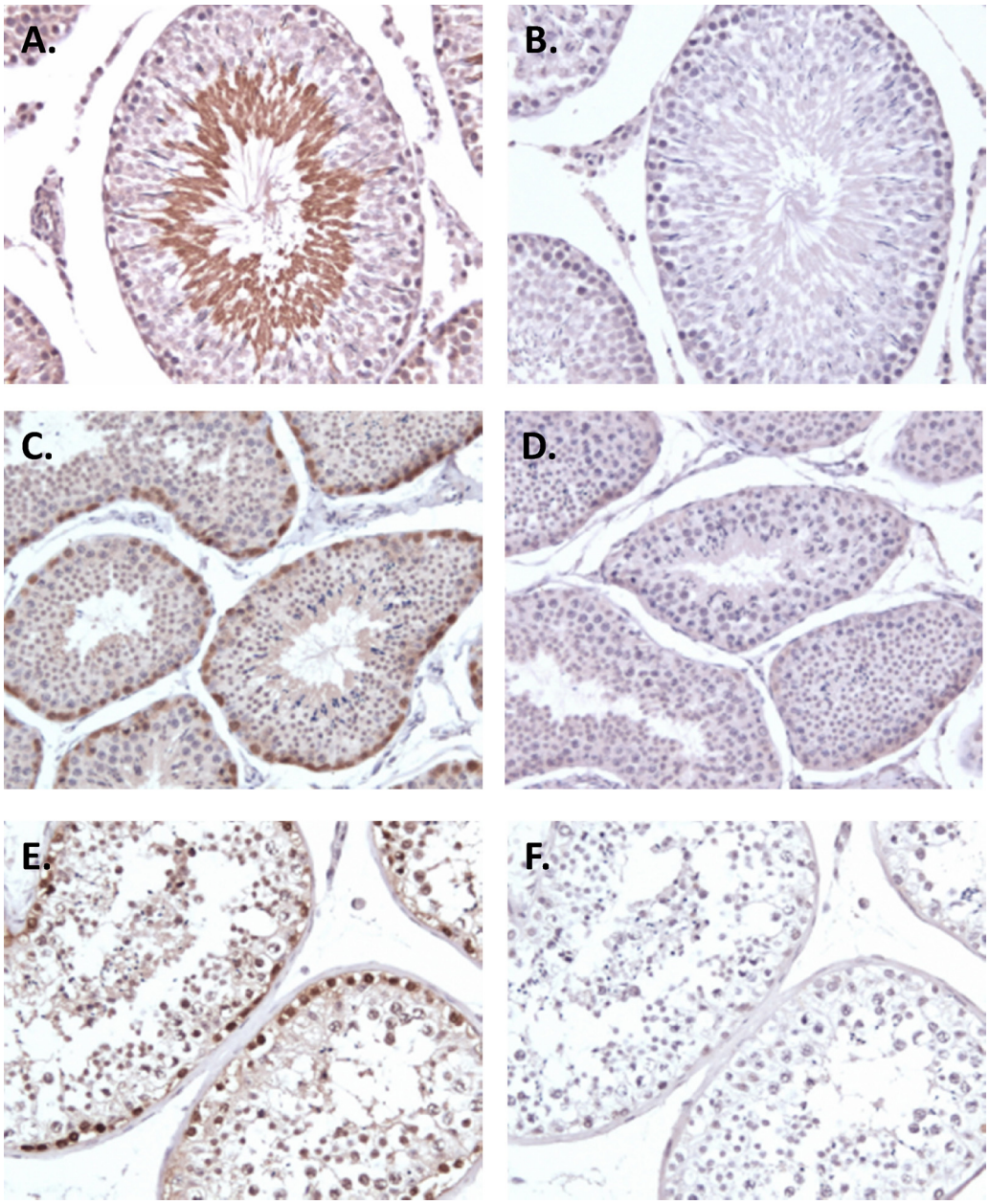

Fig. 3. Immunohistochemical analysis of MAGE-A3 expression in paraffin sections (5 μm) of testes from Sprague-Dawley rat (A and B), Cynomolgus monkey (C and D) and human (E and F) using the rabbit polyclonal antibody TA242 (dilution 1/1000). The antibody was pre-incubated with the human recMAGE-A3 protein (47 μg/ml) before use (B, D, F) or it was not pre-incubated (A, C, E). 20× magnification.

Treatment with the MAGE-A3 Cancer Immunotherapeutic was generally well tolerated; injection site reactions were limited to swollen areas around the injections sites that were observed in some rats following the MAGE-A3 Cancer Immunotherapeutic injections, although they were also noticed following injections of AS15 alone. In two other nonclinical safety studies, single or 25 repeated injections of the full human dose of MAGE-A3 Cancer Immunotherapeutic, respectively, to rabbits or Cynomolgus monkeys were also well tolerated, although significant swelling was observed in monkeys after multiple injections at the same site [36].

Treatment of female rats with the MAGE-A3 Cancer Immunotherapeutic or AS15 prior to pairing and during gestation did not affect female mating performance or fertility, embryo-fetal development or pre- and post-natal offspring survival or growth up to 25 days of age. However, when comparing the MAGE-A3 Cancer Immunotherapeutic group and the control group, we observed a statistically significant increase in the litter incidence of persistent left umbilical artery. However, this minor visceral finding is of no toxicological significance, occurred in the absence of any minor or major fetal abnormalities, and was regarded as a normal variation as the incidence was within the HCD range.

Treatment of male rats with the MAGE-A3 Cancer Immunotherapeutic or AS15 did not affect reproductive organ weights, seminology parameters or the macroscopic or microscopic appearance of male reproductive tissues, male mating performance, fertility or early embryonic development.

Repeated injections of a full human dose of the MAGE-A3 Cancer Immunotherapeutic had no effect on male fertility in Cynomolgus monkeys. *In vivo* fertility parameters (serum testosterone level,

semen production and testicular and prostate volumes) were not affected. Variations in testosterone levels were observed in all monkeys throughout the treatment and treatment-free periods and were consistent with normal physiological fluctuations [48,49].

Although MAGE-A3 immunohistochemical staining was previously demonstrated in the testes of both rat and monkey, histopathology evaluations did not reveal any evidence of toxicity to the organs of the male reproductive axis. Furthermore, in a previous toxicology study in Cynomolgus monkeys, histopathological evaluation did not reveal any toxic effects of the MAGE-A3 Cancer Immunotherapeutic to the female reproductive organs [36].

The MAGE-A3 Cancer Immunotherapeutic was immunogenic in both rats and monkeys, with all animals developing MAGE-A3-specific antibodies after 1–5 injections. MAGE-A3-specific antibodies were not detected in the sera of control animals or animals prior to receiving their first injection of MAGE-A3 Cancer Immunotherapeutic (except for one female rat in Study 1 with a very low titer). Following 3 or 5 injections of 1/5th of the human dose of MAGE-A3 Cancer Immunotherapeutic to female or male rats, respectively, and 8 injections of a full human dose to male Cynomolgus monkeys, all animals showed an induction of antibody response. The timing of the administrations was chosen to ensure exposure of the animals (adults, fetuses and pups) to the specific antibody response pre-mating and throughout the different phases of the embryo-fetal development. These data confirm that the MAGE-A3 Cancer Immunotherapeutic is immunogenic in both rats and monkeys, in line with observations from the previous repeated-dose toxicity study in monkeys [36].

The cellular response induced by AS15 has been previously assessed in monkeys [36]. In an *ex vivo* intracellular cytokine staining assay it was observed that injections of the MAGE-A3 Cancer Immunotherapeutic induced MAGE-A3 T-cell specific responses. A 2.5- to 8.0-fold increase in the median percentage of CD4+ T cells producing IL-2, TNF-α or IFN-γ cytokines was observed compared to injections of saline. CD8+ T cells producing the same cytokines were not detected. AS15 was selected as immunostimulant in the MAGE-A3 Cancer Immunotherapeutic due to its ability to induce a Th1-biased immune response and because it had the highest efficiency against the growth of MAGE-A3-expressing tumor cells when compared to other immunostimulants such as AS01, AS02 or CpG alone [52]. However, it has been previously suggested that Th1 cytokines could be unfavorable to pregnancy outcome [53]. In our study there was no evidence that maternal treatment with the MAGE-A3 Cancer Immunotherapeutic or AS15 had any adverse effect on litter data, as assessed by the pre- and post-implantation losses.

Taken together, the results of our nonclinical safety studies indicate that repeated injections of MAGE-A3 Cancer Immunotherapeutic in rats or monkeys induced immune responses without any significant indication of reproductive toxicity.

5. Conclusions

Three to five injections of 1/5th of a human dose of the MAGE-A3 Cancer Immunotherapeutic in rats or eight injections of a full human dose in Cynomolgus monkeys were well tolerated.

Treatment with MAGE-A3 Cancer Immunotherapeutic did not affect female rat mating performance or fertility, or embryo-fetal or pre- and post-natal survival, growth or development of the offspring up to 25 days of age, male mating performance (rats) or fertility parameters (rats and monkeys).

Taken together, the findings from these nonclinical safety studies suggest that repeated injections of MAGE-A3 Cancer Immunotherapeutic in rats or monkeys induced antibody immune responses and did not cause any significant reproductive toxicity.

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Conflict of interest

E. Destexhe, N. Baudson, C. Gérard, and L. Segal are employees of GSK Vaccines. N. Garçon was employee of GSK Vaccines at the time of the study. E. Destexhe, C. Gérard, L. Segal and N. Garçon declare stock ownership in the GSK group of companies. C. Gérard and N. Garçon are also inventor on patents owned by GSK group of companies. E. Grosdidier and R. Forster are employees of CiToxLab France and D. Stannard and O.K. Wilby are employees of Huntingdon Life Sciences, two CRO (Contract Research Organisation) where the safety studies have been performed on a contractual basis with GlaxoSmithKline Biologicals SA.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.12.009.

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